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Short Communication

Immunohistochemical Analysis of Bcl-2 Family Proteins in Adenocarcinomas of the Stomach

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The apoptosis-regulating proteins Bcl-2, Bax, Bcl-X, Bak, and Mcl-1 were examined by immunohistochemical methods in 48 archival specimens of adenocarcinoma of the stomach, and the results were correlated with tumor histology (intestinal* versus *diffuse pattern) and clinical stage (early-* versus *late-stage disease, ie, stages I and II* versus *stage III). Tumor cells containing immunostaining for the anti-apoptotic proteins Bcl-2, Bcl-X, and Mcl-1 were present in 26 (54%), 41 (85%), and 36 (75%) of the 48 cases evaluated, respectively, whereas immunopositivity for the pro-apoptotic proteins Bax and Bak was found in 44 (92%) and 42 (88%) specimens. Comparisons of these immunostaining results with tumor histology revealed statistically significant differences for Bax (P = 0.03), Bcl-X (P = 0.003), and Mcl-1 (P = 0.005), which were all more frequently immunopositive for tumors with an intestinal than a diffuse histological pattern (χ^2 analysis). In addition, the percentage of immunopositive tumor cells was significantly higher for Bcl-X (62 ± 6% versus 45 ± 6%, mean ± SE; P = 0.01) and for Mcl-1 (48 ± 6% versus 30 ± 6%; P = 0.04) in tumors with intestinal versus diffuse histology (unpaired t-test). In contrast, the percentage of Bcl-2-immunopositive tumor cells was higher in tumors with diffuse histology compared with intestinal (32 ± 5% versus 12 ± 5%; P = 0.01), whereas the percentages of Bax- and Bak-immunopositive tumor cells were not significantly different between these two histological types. In 34 specimens, residual normal gastric epithelial cells (foveolar cells) were present for direct comparisons of immunointensity with tumor cells. The immunointensity for the Bcl-2, Bcl-X, and Mcl-1 proteins was stronger in tumor cells compared with normal foveolar cells in 7 (21%), 15 (44%), and 8 (24%) of 34 cases, respectively, whereas the immunointensity of the pro-apoptotic proteins Bax and Bak was reduced compared with normal cells in 8 (24%) and 24 (71%) cases. Immunointensity, however, did not correlate with histology. Clinical stage was not significantly associated with the presence or absence of immunopositive tumor cells, the percentage of immunopositive cells, or immunointensity. Taken together, these results establish for the first time that several Bcl-2 family proteins are expressed in gastric adenocarcinomas and suggest that the repertoire of these proteins may differ depending on the histological type. The findings therefore support the notion that the intestinal and diffuse types of gastric cancer arise at least in part through different mechanisms. (Am J Pathol 1996, 149:1449–1457)

Tumors can arise from alterations in the activity of genes that result in accelerated rates of cell division,

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decreased rates of cell death, or both (reviewed in Refs. 1–4). Among the genes that regulate cellular life span are members of the *bcl-2* family. The *bcl-2* gene was first discovered by virtue of its involvement in the t(14;18) chromosomal translocations that commonly occur in non-Hodgkin's B-cell lymphomas.[5] Overexpression of the *bcl-2* gene prevents or delays normal cell turnover caused by programmed cell death, thus prolonging cellular life span and contributing to the clonal expansion of neoplastic cells.[6,7] High levels of the Bcl-2 protein can also protect cancer cells from apoptotic cell death induced by a wide variety of stimuli and insults, including radiation and nearly all chemotherapeutic drugs, as well as loss of attachment to extracellular matrix proteins (an issue of relevance to metastasis) and attack by cytolytic T cells (reviewed in Refs. 3, 8, and 9). Thus, inappropriate increases in the production of the Bcl-2 protein may play a potentially important role in the pathogenesis and progression of human tumors and in their responses to therapeutic interventions.

Although its biochemical mechanism of action remains enigmatic, the Bcl-2 protein functions at least in part through its interactions with other proteins, including several homologous proteins that constitute the Bcl-2 protein family. Some of the members of this protein family are blockers of cell death such as Mcl-1 and the most abundant form of the Bcl-X protein (Bcl-X_L), whereas others are promoters of cell death such as Bax and Bak.[10–15] With the exception of Bcl-2, relatively little is known presently about the expression of these apoptosis-regulating proteins in human cancers, including adenocarcinomas of the stomach.

Among all cancers in the United States, adenocarcinoma of the stomach ranks 14th in the frequency of new cases per year and 7th in cancer-related deaths, with nearly 23,000 new cases and almost 15,000 deaths per year.[16] Gastric cancer, however, is among the most common types of malignancy in some parts of the world, such as in Japan and in parts of China, South America, and Scandinavia, where it is associated with ingestion of foods that may contain relatively high amounts of nitrosamines.[17–19] Gastric cancers are generally poorly responsive to chemotherapy and radiotherapy, suggesting that these tumors are intrinsically resistant to the apoptosis-inducing effects of anticancer drugs and x-irradiation. The 5-year survival for gastric cancer is generally <10%, a statistic that has not changed appreciably in the past 60 years.

Adenocarcinomas of the stomach occur in two major histological subtypes, intestinal and diffuse.[17–19] The intestinal variant is thought to arise from gastric mucosa cells that have undergone intestinal metaplasia. This more differentiated form of gastric cancer is more common in high-risk populations and often seen in the setting of chronic gastritis, recently attributed to infection with *Helicobacter pylori* in most cases.[18,19] It generally occurs after age 50 and displays an approximately 2:1 male predominance. The diffuse form is hypothesized to arise *de novo* from native gastric epithelial cells and is poorly differentiated in general. It constitutes roughly one-half of gastric cancers in the United States, tends to occur earlier in life than the intestinal variant, and has no predilection for males. Taken together, therefore, these histological features and epidemiological associations suggest different mechanisms for the pathogenesis of the intestinal and diffuse forms of gastric cancer.

In this report, we used immunohistochemical methods to characterize for the first time the expression of several *bcl-2* family genes in gastric adenocarcinomas. Differences in the expression of some *bcl-2* family genes in the intestinal and diffuse variants support the hypothesis that these two histological subtypes of gastric cancer can arise, at least in part, through different mechanisms.

Materials and Methods

Patient Specimens

Gastric cancer specimens were derived from patients who presented to the Hospital of the University of Cincinnati as part of SWOG study 9008. The histological diagnosis was confirmed by expert pathologists (C. Fenoglio-Preiser and G. Stemmerman), classified as intestinal (n = 25), diffuse (n = 20), or mixed (n = 3), and the patients were staged by the TNM system (n = 10 stage I and II; n = 38 stage III).[17]

Immunostaining Methods

Paraffin-embedded tumor specimens (n = 48) that had been fixed in neutral-buffered formalin were sectioned (5 μm) and immunostained using anti-peptide polyclonal antisera specific for the Bcl-2, Bax, Bcl-X, Bak, and Mcl-1 proteins exactly as described in detail previously.[20–25] The specificity of all of these antibody reagents has been demonstrated based on comparisons with preimmune serum, peptide competition experiments, and immunoblot analysis of tissue extracts and cell lines, including transfected cell lines engineered to ectopically express the specific antigens.[20–25] Immunodetection was achieved by an

avidin-biotin horseradish-peroxidase-based colorimetric method using 3,3′-diaminobenzidine as the chromogen, followed by light counterstaining with hematoxylin. The specificity of the immunostaining was confirmed in selected cases by performing the immunostaining procedure using preimmune sera (not shown) or antisera preadsorbed with the immunizing peptide (for example, see Figure 1C).

The immunostaining results were scored as negative if no immunopositive tumor cells were present, but immunopositive non-neoplastic cells were available in the same section as an internal positive control (normal gastric cells, lymphocytes, smooth muscle cells, etc). All 48 cases contained internal positive control cells for all five antibodies. In addition, the approximate percentage of immunopositive tumor cells was estimated from a minimum of two representative fields. The intensity of the immunostaining in tumors and normal gastric epithelial cells was directly compared side by side in the same sections, in those specimens that contained residual normal gastric epithelial tissue (n = 34). The tumor immunointensity was scored as less than (−1), equal to (0), or greater than (+1) the normal foveolar cells. In addition, the intensity of immunostaining in all 48 cases was arbitrarily scored as negative (0), weak/moderate (1+), or strong/intense (2+).

Statistics

Analysis of data was accomplished using the JMP Statistics software package (SAS Institute). Immunointensity was compared with histology and clinical stage by χ^2 analysis, using a Pearson test with either a 2 × 2 or 2 × 3 matrix. The percentage of immunopositive tumor cells was compared with histology and stage using an unpaired *t*-test. For tumors with mixed diffuse and intestinal patterns, only the diffuse component was scored and the case was evaluated as diffuse pattern. For correlations with clinical stage, patient data were dichotomized into early (stages I and II) and late (stage III) subgroups.

Results

All 48 cases of gastric cancer were successfully immunostained for all five antibodies. Immunopositive normal or tumor cells were present in all specimens, verifying adequate preservation of the epitopes. The immunostaining was also specific, in that only cytosolic immunostaining was seen, sometimes in a granular pattern typical of Bcl-2 family proteins, which are usually associated with cytosolic organelles such as mitochondria and endoplasmic reticulum.[26] In the adjacent normal stomach tissue, moderate to strong intensity Bcl-2 immunostaining was typically present in the mucous cells lining the gastric pits (foveolar cells; Figure 1A). This Bcl-2 immunoreactivity was often oriented toward the luminal side of the cells, particularly in the cells located deeper in the gastric pits. Parietal and chief cells were weakly Bcl-2 immunopositive. In contrast to Bcl-2, immunostaining for Bax was generally weak in normal gastric mucosal cells, although the cells located at the surface of the stomach did often contain moderate intensity Bax immunoreactivity. Interestingly, intense Bax immunoreactivity resided in the enteroendocrine cells scattered throughout the gastric neck and base region of the glands (Figure 1B). Bcl-X immunostaining was distributed unhomogeneously in the foveolar cells, with the immunointensity ranging from essentially negative to moderate in intensity (Figure 1D). Bak immunostaining was present at moderate to strong intensity in the foveolar cells. The parietal cells contained intense Bak immunoreactivity (Figure 1, E and F). Moderate intensity Mcl-1 immunoreactivity was homogeneously present within the foveolar cells throughout the gastric pits (Figure 1, G and H). The stromal tissue surrounding the gastric glands was mostly immunonegative for Bcl-2, Bcl-X, Mcl-1, and Bax, but occasional fibroblasts stained for Bcl-2 and Bak. The results of this immunohistochemical analysis of Bcl-2, Bcl-X, Bax, Mcl-1, and Bak were essentially identical to previous reports in which the expression of these proteins was examined in normal stomach tissue.[20,24,27,28]

Within the cancerous portions of the tissue sections, immunopositive tumor cells were found for Bcl-2, Bax, Bcl-X, Bak, and Mcl-1 in 26 (54%), 44 (92%), 41 (85%), 42 (88%), and 36 (75%) of the 48 cases, respectively. As summarized in Table 1, comparisons of the presence or absence of immunopositive tumor cells with intestinal *versus* diffuse histology by χ^2 analysis revealed statistically significant associations of Bax, Bcl-X, and Mcl-1 with intestinal histology. For example, 25 of 25 (100%) of the intestinal cases were Bax positive compared with 19 of 23 (83%) of the tumors with diffuse histology ($P = 0.03$). Similarly, 25 of 25 (100%) intestinal-type tumors were Bcl-X immunopositive, whereas only 16 of 23 (70%) diffuse histology specimens contained Bcl-X-positive tumor cells ($P = 0.003$). Immunopositivity for Mcl-1 was also associated more frequently with intestinal histology (23 of 25 (92%) intestinal *versus* 13 of 23 (57%) diffuse; $P = 0.005$), but Bcl-2 and Bak immunopositivity were not significantly associated with either intestinal or diffuse histology.

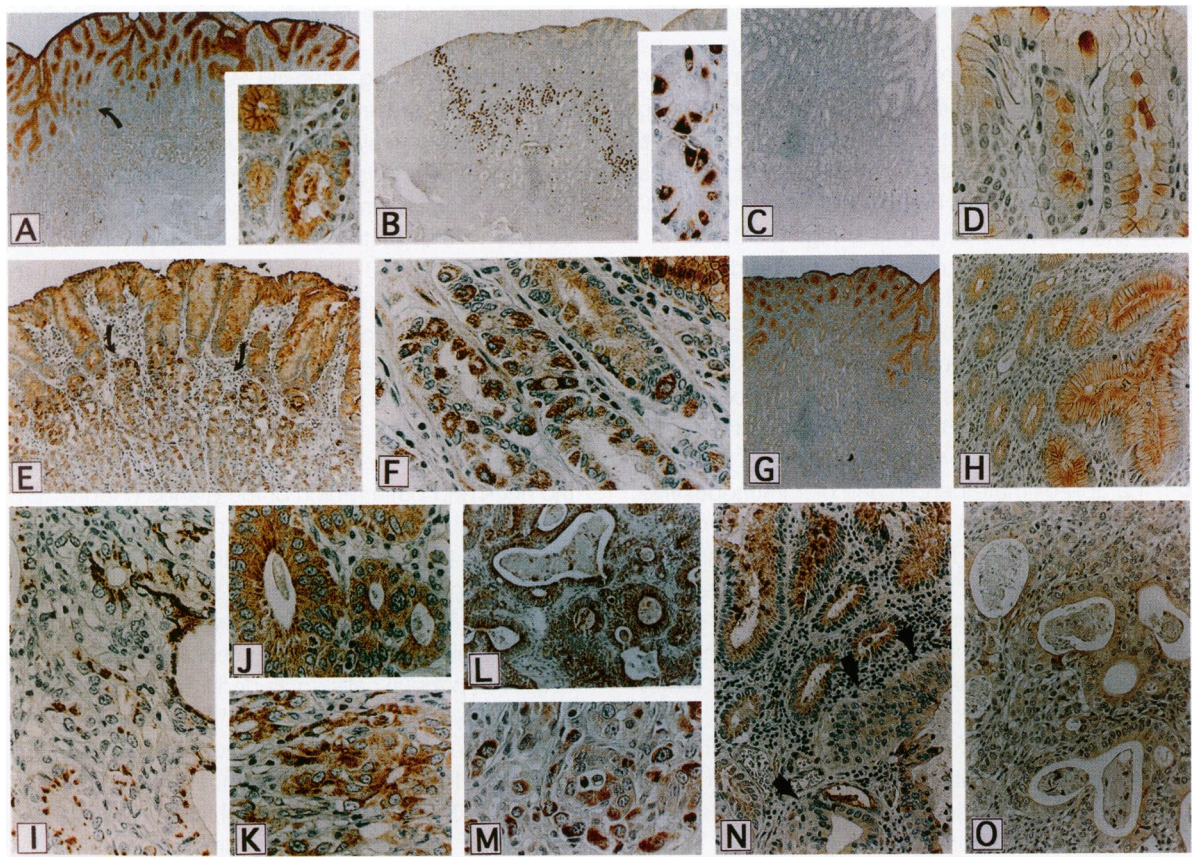

Figure 1. *Examples of immunostaining results for Bcl-2, Bax, Bcl-X, Bak, and Bak in normal gastric mucosa and gastric cancers.* **A:** *Bcl-2 immunostaining in normal gastric mucosa, showing homogeneous moderate to strong immunoreactivity in mucosa cells that line the upper and middle portions of the gastric pits and surface of the stomach, ie, foveolar cells. Magnification, × 70.* **Arrow** *indicates area shown at higher magnification (×400) in the* **inset**, *illustrating foveolar cells in a cross section through a gastric pit.* **B:** *Bax immunostaining in an area of normal stomach, showing relatively weak immunoreactivity in most mucous cells with moderate intensity immunostaining in the epithelial cells located at the surface of the stomach. Magnification, × 70. Intense Bax immunoreactivity is seen in cells scattered throughout the neck and bases of the gastric glands. The* **inset** *represents a higher-magnification view (×400) of these intensely immunostained enteroendocrine (APUD) cells in the gastric neck.* **C:** *Peptide competition experiment using Bax antiserum that was preadsorbed with Bax peptide; the tissue section was derived from the same case as in* **B**. *Magnification, × 100.* **D:** *Bcl-X immunostaining in normal foveolar cells at surface of stomach, showing heterogeneous immunostaining, with most cells containing only weak or no immunoreactivity but occasional cells exhibiting moderate intensity immunostaining. Magnification, ×400.* **E:** *Bak immunostaining in normal stomach (fundus region), showing moderate to strong immunoreactivity in foveolar cells lining gastric pits and luminal surface of stomach and intense immunostaining in parietal cells located in neck and bases of the gastric glands* (**arrows**). *Magnification, ×150.* **F:** *Higher-magnification view (×400) of same section shown in* **E**, *showing parietal cells with strong Bak immunostaining.* **G** *and* **H:** *Mcl-1 immunostaining in normal stomach, showing weak to moderate intensity immunostaining in foveolar cells. Magnification, × 70 and ×200, respectively.* **I:** *Bcl-2 immunostaining in gastric tumor with combinations of diffuse (poorly differentiated) and intestinal (well differentiated) histology, showing intense Bcl-2 immunostaining localized to supranuclear cytosolic structures in cells that form glandular structures resembling small intestine but little or no immunoreactivity in tumor cells diffusely infiltrating through surrounding stroma. Magnification, ×200.* **J** *and* **K:** *Bax immunostaining in gastric adenocarcinoma with intestinal* (**J**) *and diffuse* (**K**) *histology, showing that tumor cells uniformly contain strong Bax immunoreactivity. Magnification, ×400.* **L** *and* **M:** *Bcl-X immunostaining in gastric cancer with mostly intestinal-type histology* (**L**) *but also with a diffuse component* (**M**), *demonstrating strong Bcl-X immunoreactivity in most of the tumor cells. Magnification, ×150 and ×400, respectively.* **N:** *Bak immunostaining in a case of gastric cancer, comparing normal moderate to strong intensity Bak immunoreactivity in adjacent normal mucosa* (**top**) *with weaker Bak immunostaining in tumor* (**bottom**; **arrows**). *Magnification, ×200.* **O:** *Mcl-1 immunostaining in the same case of gastric cancer as in* **J**, *showing weak to moderate intensity immunoreactivity in tumor cells located both in the glandular structure and infiltrating through stroma, similar to normal epithelium. Magnification, ×200. The intensity of Mcl-1 immunostaining is slightly stronger in well differentiated tumor cells, particularly at the luminal aspect of the cells forming glandular structures.*

To explore the possibility that the presence of even a few immunopositive tumor cells might skew the results, these statistical correlations were repeated using ≤10% immunostained tumor cells as an arbitrary cut-off for designating immunopositivity for the cases. As before, however, Bax, Bcl-X, and Mcl-1 were significantly associated with intestinal histology ($P = 0.03$, 0.003, and 0.005, respectively), whereas Bcl-2 and Bak were not associated with either histological subtype of tumor (not shown). No significant correlations with early- *versus* late-stage disease were found, regardless of whether we used the presence or absence of immunopositive tumor cells or <10% *versus* ≥10% immunopositive tumor cells to dichotomize the data.

Table 1. *Comparisons of Presence or Absence of Immunopositive Tumor Cells with Histology*

Histology	Protein				
	Bcl-2	Bax	Bcl-X	Bak	Mcl-1
Intestinal (n = 25)	13 (52%)	25 (100%)	25 (100%)	23 (92%)	23 (92%)
Diffuse (n = 23)	13 (57%)	19 (83%)	16 (70%)	19 (83%)	13 (57%)
Total (n = 48)	26 (54%)	44 (92%)	41 (85%)	42 (88%)	36 (75%)
(*P* value)	NS	0.03	0.003	NS	0.005

The proportion of tumors with intestinal (n = 25) or diffuse (n = 23) histology (and percentages of cases) that contained Bcl-2-, Bax-, Bcl-X-, Bak-, or Mcl-1-immunopositive tumor cells is indicated. χ^2 analysis was used to compare results for intestinal and diffuse groups. NS, nonsignificant ($P > 0.05$).

The percentage of immunopositive tumor cells was estimated and correlated as a continuous variable with intestinal *versus* diffuse histology and with early *versus* late clinical stage. As summarized in Figure 2, the percentage of Bcl-2-positive tumor cells was significantly higher among the diffuse histology tumors compared with the intestinal type (32 ± 5% diffuse *versus* 12 ± 5% intestinal, mean ± SE; $P = 0.01$ by unpaired *t*-test). Conversely, the percentage of Bcl-X-positive and the percentage of Mcl-1-positive tumor cells was significantly higher in the intestinal histology subgroup compared with the diffuse variant. Specifically, the mean (± SE) percentage of Bcl-X-immunopositive tumor cells among the intestinal cases was 68 ± 6% compared with 45 ± 6% for the diffuse histology specimens ($P = 0.01$). Similarly, the mean percentage of Mcl-1-immunopositive tumor cells was 48 ± 6% compared with 30 ± 6% for the intestinal and diffuse cases, respectively ($P = 0.04$). The percentages of Bax- and Bak-immunopositive cells were somewhat higher on average among the intestinal histology cases compared with the diffuse type (72 ± 6% intestinal *versus* 62 ± 6% diffuse for Bax; 63 ± 6% intestinal *versus* 53 ± 6% diffuse for Bak), but these slight differences were not statistically significant (Figure 2).

Figure 2. *Comparison of percentages of Bcl-2, Bax, Bcl-X, Bak, and Mcl-1 immunopositive tumor cells in gastric cancers with intestinal* versus *diffuse histology. The percentages of immunopositive tumor cells were estimated after immunostaining using antibodies specific for Bcl-2, Bax, Bcl-X, Bak, or Mcl-1. Comparisons were made for tumors with intestinal (I)* versus *diffuse (D) histological patterns, and statistical significance was determined by unpaired* t*-test. NS, nonsignificant (P > 0.05).*

The percentage of Bcl-2-, Bax-, Bcl-X-, Bak-, or Mcl-1-immunopositive tumor cells did not correlate with clinical stage. It may be of interest, however, that all tumors that contained relatively low percentages of Bax-immunopositive cells (<35% Bax immunopositive) were derived from patients with advanced stage disease (7 of 7; not shown). Otherwise, no trends in the percentage of immunostained cells and clinical stage were noted.

In 34 of the 48 gastric cancer specimens evaluated here (15 intestinal and 19 diffuse), residual normal gastric mucosal epithelium was present within the same sections along with tumor, thus permitting direct comparisons of the intensity of the immunostaining in tumors with normal cells (Table 2A). These comparisons were made with the surface epithelial mucous cells (foveolar cells) that line the gastric pits, a population of cells with rapid turnover rates (~5 days) from which gastric cancers are thought to derive.[17–19] All five of the Bcl-2 family proteins examined here, Bcl-2, Bax, Bcl-X, Bak, and Mcl-1, were expressed in these normal cells, although at different relative intensities as mentioned above (see Table 2 footnote for details). The epithelial cells located below the gastric pits in the gastric neck region as well as the parietal and chief cells in the bases of the gastric glands were ignored for the purposes of comparisons of immunointensity in normal and tumor cells. These epithelial and secretory cells located in the lower portions of the gastric glands have very slow turnover rates (~1 to 2 years) and therefore are unlikely to be a source of gastric carcinomas.[17–19]

The intensity of Bcl-2 was greater than normal epithelium in 7 (21%), equivalent to normal in 7 (21%), and less than normal in 20 (59%) of the tumors. Thus, although elevations in Bcl-2 have been associated with the pathogenesis of lymphoma and progression of prostate cancer,[29,30] Bcl-2 expression was more frequently reduced than elevated in gastric cancers (see Figure 1I, for example). Similarly, although Mcl-1 has been shown to function as an inhibitor of apoptosis,[12] the intensity of Mcl-1

Table 2. *Assessment of Immunointensity in Gastric Adenocarcinomas*

	Bcl-2	Bax	Bcl-X	Bak	Mcl-1
A. Normal *versus* Tumor (n = 34)					
T < N*	20 (59%)	8 (24%)	9 (26%)	24 (71%)	16 (47%)
T = N	7 (21%)	10 (29%)	10 (29%)	7 (21%)	10 (29%)
T > N	7 (21%)	16 (47%)	15 (44%)	3 (9%)	8 (24%)
B. Arbitrary Scale (n = 48)					
0	22 (46%)	4 (8%)	7 (15%)	6 (13%)	12 (25%)
1	17 (35%)	24 (50%)	23 (48%)	33 (68%)	34 (71%)
2	9 (19%)	20 (42%)	18 (38%)	9 (19%)	2 (4%)

A: The intensity of immunostaining found in immunopositive tumor cells was compared directly with adjacent normal foveolar cells: T < N, tumor intensity less than normal; T = N, tumor intensity equivalent to normal; T > N, tumor intensity greater than normal. In cases for which immunoreactivity was unhomogeneous in normal epithelium (eg, Bcl-X, for which only ~50 to 70% of cells were immunopositive) and in tumors, the immunonegative cells were ignored for purposes of scoring. Moreover, the average immunointensity of normal immunopositive cells was scored against average intensity of immunopositive tumor cells, thus avoiding extremes of low and high intensity.

*Includes cases in which no immunopositive tumor cells were found (i.e. 0% immunopositive tumor cells) (Bcl-2, 22; Bax, 4; Bcl-X, 7; Bak, 6; Mcl-1, 12). These cases were all scored as T < N.

B: Immunointensity was scored using an arbitrary three-point system as negative (0), weak to moderate (1+), and strong to intense (2+). A score of 0 indicates no immunopositive cells were present in the tumor. The immunointensity in the normal foveolar cells was as follows: Bcl-2, 2+; Bax, 1+; Bcl-X, 1+; Bak, 2+; and Mcl-1, 1+.

immunostaining was reduced compared with normal gastric epithelium in 16 (47%) of 34 cases, whereas Mcl-1 immunointensity was equal to normal in 10 (29%) and greater than normal in 8 (24%) specimens (Figure 1O). In contrast, Bcl-X immunointensity was frequently elevated in tumors compared with normal epithelium, with Bcl-X intensity greater than normal in 15 (44%) of 34 cases (Figure 1, L and M), equivalent to normal in 10 tumors (29%), and less than normal in 9 specimens (26%; Table 2A). The intensity of immunostaining for the pro-apoptotic protein Bax was reduced compared with normal foveolar cells in 8 (24%) of 34 cases. Surprisingly, however, Bax immunointensity was more frequently higher than normal (16 of 34, 47%) in the gastric cancers examined here (see Figure 1, B, J, and K). In contrast, the immunointensity of another pro-apoptotic protein Bak was commonly reduced in gastric cancers compared with normal foveolar cells (24 of 34, 71%). In the case of Bak, immunointensity was elevated compared with normal in only 3 (9%) of 34 specimens and equivalent to normal in 7 (21%) tumors (Table 2A; Figure 1N). The immunointensity data presented in Table 2A included samples in which no immunopositive cells were found (ie, 0% immunostained tumor cells), in which case the tumors were scored as intensity less than normal. No significant correlations of immunointensity with histology (diffuse *versus* intestinal) or clinical stage (early *versus* late) were found.

In addition to scoring immunointensity relative to adjacent normal foveolar cells, the intensity of Bcl-2, Bax, Bcl-X, Bak, and Mcl-1 immunostaining was scored on an arbitrary three-point scale (0, 1+, 2+) for all 48 tumors. Overall, these data corroborated the findings obtained by direct comparisons of normal and tumor cells (see Table 2B for details). For example, Bcl-X immunostaining was strong or intense (2+) in 18 (37%) of 48 tumors, whereas Bcl-X immunointensity was typically only low to moderate (1+) in normal gastric epithelium. In addition, Bak immunostaining was absent or present at only low to moderate intensity in 39 (81%) of 48 tumors, whereas the intensity of Bax immunostaining was usually strong to intense (2+) in normal foveolar cells (Table 2B). Again, however, these immunointensity data failed to correlate with either histology or clinical stage (not shown).

Discussion

In this report, we characterized for the first time the expression of several Bcl-2 family proteins in gastric adenocarcinomas. All five of the Bcl-2 family proteins examined here (Bcl-2, Bax, Bcl-X, Bak, and Mcl-1) were frequently expressed in gastric cancers. However, the percentage of immunopositive cells was highly variable for all five of these proteins, implying that genetic alterations or environmental stimuli (such as from growth factors or cell adhesion molecules) may control the production of these apoptosis-regulating proteins in complex ways. It has been hypothesized that the intestinal (well differentiated) and diffuse (poorly differentiated) variants of gastric cancer arise at least in part as a consequence of different genetic alterations (reviewed in Ref. 31). For example, the intestinal variants of gastric adenocarcinomas commonly contain K-*ras* mutations, c-*erb*B-2 gene amplification, loss of heterozygosity (LOH) in the region of the *APC* gene, and LOH at the *DCC* gene locus, whereas the diffuse variants of

these tumors more often contain c-*met* and K-*sam* gene amplification, LOH at the p53 gene, and loss of expression of cadherins and catenins. Interestingly, LOH of the *bcl-2* gene has been reported in 43% of intestinal histology gastric cancers but was not seen in diffuse histology tumors.[32] As the *bcl-2* gene resides near the *DDC* gene on chromosomal band 18q21, it is likely that LOH of *bcl-2* represents a by-product of the genetic deletions that lead to inactivation of the *DDC* tumor suppressor gene in gastric cancers, rather than a true step in the progression of the intestinal form of gastric cancer. In addition, another tumor suppressor gene involved in pancreatic cancer, *DCP4*, which may play a role in transforming growth factor-β receptor signal transduction, has recently been identified at 18q21.[33] Consistent with the LOH of the *bcl-2* gene reported in well differentiated gastric cancers, we found that the percentage of Bcl-2-immunopositive tumors cells was generally lower among intestinal histology tumors compared with those with predominantly diffuse histology ($P = 0.01$; Figure 1). It remains to be determined, however, to what extent this lower percentage of Bcl-2-immunopositive tumor cells may be functionally related to loss of one copy of the *bcl-2* gene among tumors with intestinal histology.

In contrast to the tendency of tumors with intestinal histology to contain lower percentages of Bcl-2-immunopositive cells, the percentage of Bcl-X- and Mcl-1-immunopositive tumor cells was generally higher among these tumors compared with diffuse gastric adenocarcinomas ($P = 0.01$ and $P = 0.04$, respectively; Figure 2). The proportion of cases of gastric cancer that contained Bcl-X- or Mcl-1-immunopositive tumor cells was also higher for those with intestinal than diffuse histology ($P = 0.003$ and $P = 0.005$, respectively; Table 1). Given that Mcl-1 and the most commonly produced form of Bcl-X, the Bcl-X_L protein, are known to suppress cell death,[11,12] one implication of these findings is that Mcl-1 or Bcl-X may substitute for Bcl-2 in tumors with intestinal histology. Thus, functional redundancy among the anti-apoptotic members of the Bcl-2 protein family may allow these tumor cells to tolerate the absence of Bcl-2 by producing Bcl-X_L or Mcl-1 instead. In this regard, although the intensity of Bcl-2 immunostaining compared with adjacent normal gastric epithelium was more often reduced than elevated (59% reduced *versus* 21% elevated; Table 2A), Bcl-X immunointensity was more frequently elevated than reduced in the gastric cancers examined here (44% elevated *versus* 26% reduced; Table 2A). A similar phenomenon has been observed for colorectal adenocarcinomas,[25] implying that elevated levels of Bcl-X protein may represent a common event in the pathogenesis or progression of epithelial malignancies of the gastrointestinal tract. One caveat with the Bcl-X immunostaining results is that antibodies are not yet available that discriminate among the various forms of the Bcl-X protein that can potentially be produced as a result of alternative mRNA splicing events.[11] However, in all normal tissues surveyed throughout the body and in all tumors examined to date, by far the most abundant form of the Bcl-X protein found has been the anti-apoptotic Bcl-X_L protein, and very little or none of the pro-apoptotic Bcl-X_S protein was detected.[20,34–36]

The reduced intensity of Bak immunostaining compared with adjacent normal gastric epithelium seen in 71% of the tumors evaluated here that contained residual normal epithelium for direct comparisons (Table 2A) implies that decreases in the levels of this pro-apoptotic protein occur frequently in gastric cancers. Interestingly, reduced Bak immunointensity has also been observed in a high percentage (~90%) of colorectal cancers, when compared with adjacent normal colonic epithelium.[25] In contrast, Bax immunointensity was more often elevated than reduced in gastric cancers (47% elevated *versus* 24% reduced; Table 2A), which appears to defy *a priori* expectations in view of the evidence that 1) Bax is a promoter of apoptosis and 2) tumor cells gain a selective growth advantage relative to their normal counterparts at least in part by reducing their tendency to undergo apoptosis. Recent studies of *bax*-deficient (knock-out) mice, however, suggest that the Bax protein can either promote or suppress cell death, depending on the cellular context within which it is expressed.[37] It will be of interest therefore to contrast the function of Bax in gastric cancers, in which Bax immunostaining is often elevated, with other tumors in which Bax immunostaining has been shown to be reduced, such as adenocarcinomas of the breast.[23,38]

Notwithstanding the increased intensity of Bax immunostaining seen in some tumors, it should be noted that a few of the cases of gastric carcinoma examined here (4 of 48, 8%) contained no Bax-immunopositive tumors, and in 8 cases (17%), over one-half of the tumor cells were Bax immunonegative. As the normal gastric epithelial cells that line the gastric pits are generally all Bax immunopositive, the implication is that reductions in *bax* expression have occurred in these tumor cells. Indeed, if one assumes that the presence of any Bax-immunonegative tumor cells represents a reduction in Bax relative to normal epithelium where ~100% of the cells are typically Bax positive, then it could be argued that

most (47 of 48, 98%) of the gastric cancers examined here had evidence of loss of Bax expression. It remains to be determined, however, whether the absence of immunodetectable Bax protein endows these tumor cells with a selective survival advantage relative to the Bax-positive cells. If true, then a testable hypothesis that follows from these observations is that, during progression of tumors, there should occur a gradual enrichment for Bax-negative cells. Moreover, given the important role that has been suggested for Bcl-2 family proteins as determinants of intrinsic chemoresistance (reviewed in Refs. 3 and 8), it will be of interest in future studies to compare the relative percentages and intensity of immunostaining for Bax as well as the other Bcl-2 family proteins examined here in gastric tumors before and after chemotherapy, searching for evidence of preferential survival of tumor cells that have either down-regulated the pro-apoptotic proteins Bax and Bak or that have up-regulated the levels of the anti-apoptotic proteins Bcl-2, Bcl-X, and Mcl-1.

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